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Short Communication

Even juvenile leaves of tobacco exhibit programmed cell death

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Abstract Although the relationship between leaf senescence and programmed cell death (PCD) has been actively researched, no research had been carried out previously on juvenile and young leaves. We showed evidence of PCD (DNA laddering by agarose gel electrophoresis and DNA fragmentation by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assays) for juvenile and young leaves of *Nicotiana tabacum*. Additionally, confocal laser scanning microscopy of nuclei in protoplasts from juvenile leaves clarified the beginning stages of nuclear fragmentation. We concluded that even juvenile leaves of tobacco exhibit PCD and that tobacco leaves grow while undergoing PCD from the initial stages of development until senescence.

Key words: Confocal laser scanning microscopy, juvenile leaves, *Nicotiana tabacum*, programmed cell death, TUNEL.

PCD has been defined in animal systems as a type of cell death that is a normal part of an organism's life cycle (Ellis et al. 1991), playing essential roles in the formation of specific tissues during development (Fukuda 2000). Many recent studies in plants have revealed that PCD occurs in some developmental processes, including anther development, sex determination, tracheary element differentiation, germination within aleurone layers of monocotyledons, endosperm degeneration, nucellus degeneration, leaf shape remodeling, leaf senescence, petal senescence and early senescence (Wang et al. 1999; Hao et al. 2003; Fukuda 1997; Fath et al. 2000; Young et al. 1997; Gunawardena et al. 2004; Cao et al. 2003; Orzaez and Granell 1997; Wagstaff et al. 2003).

PCD in plants is often detected by chromatin condensation, nuclear fragmentation, DNA fragmentation, and DNA laddering, which indicates internucleosomal fragmentation of DNA. DNA laddering is one of the easiest PCD markers to detect and is used in numerous systems (Wagstaff et al. 2003). TUNEL assays are used for *in situ* detection of DNA fragmentation (Mittler and Shulaev 2004).

A relationship between leaf senescence and PCD was reported in naturally senescing leaves from five tree species by Yen and Yang (1998). According to them, PCD is involved in the complex process of leaf senescence, as shown by DNA laddering by agarose gel electrophoresis and DNA fragmentation by TUNEL assay. Simeonova et al. (2000) studied leaf senescence in *Ornithogalum virens* and *Nicotiana tabacum* using the comet assay technique in order to detect early DNA damage prior to chromatin condensation, and they suggested that PCD is involved in leaf senescence in grass species. Thus, PCD may be ubiquitous in plant senescence. Additionally, the study of genes concerned with leaf senescence has been carried out actively. Senescence-associated genes (SAGs), *ore1*, *ore3*, and *ore9*, were identified during leaf senescence (Hinderhofer and Zentgraf 2001; Woo et al. 2004). Wagstaff et al. (2003) detected features of PCD such as DNA laddering in younger petals of Alstroemeria than other studies (Wagstaff et al. 2001; Wagstaff et al. 2002). PCD associated with shape remodeling of juvenile leaves has been detected in the lace plant and in *Monstera obliqua* (Gunawardena et al. 2004; Gunawardena et al. 2005). As mentioned above, leaf senescence has been actively researched, but studies on expression of PCD in juvenile leaves have not been carried out in tobacco leaves unaccompanied by shape remodeling of leaves. In our present study, leaves of *N. tabacum* were examined from the viewpoint of PCD, from early developmental stages to senescence.

N. tabacum L. cv. Red Russian was used as plant materials. Leaf samples were obtained from the seedlings with more than 10 leaves. Plants were grown in a greenhouse (28–36°C) of the School of Agriculture, Ibaraki University (Japan). Leaf development was divided into five stages for convenience (Figure 1). The youngest leaves, 2–5 cm in length, were defined as Stage

Abbreviations: CTAB, cetyltrimethylammonium bromide; DAPI, 4′-6-diamino-2-phenylindole dihydrochloride; PCD, programmed cell death; TUNEL, TdT (TdT: terminal deoxynucleotidyl transferase)-mediated dUTP nick-end labeling).

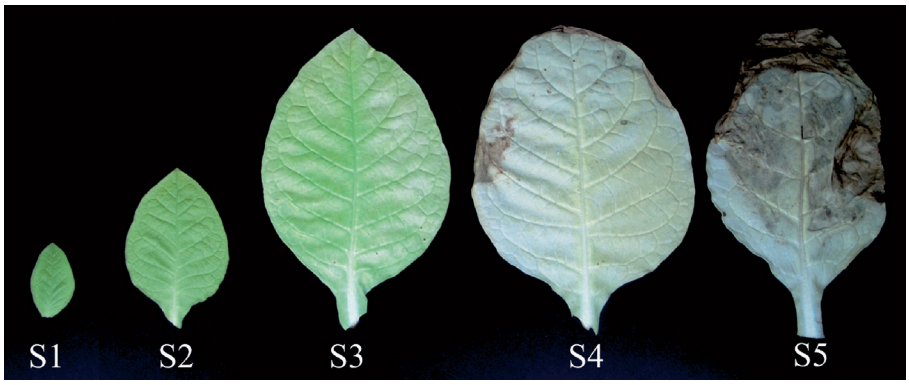

Figure 1. Stages of leaf development in *Nicotiana tabacum*. The youngest leaves of 2–5 cm in length were defined as Stage 1 (S1). The upper leaves longer than 5 cm were defined as Stage 2 (S2). Fully expanded leaves were defined as Stage 3 (S3). The basal leaves that began to become yellow were defined as Stage 4 (S4). The leaves that became fully yellow or brown were defined as Stage 5 (S5).

1. The upper leaves longer than 5 cm and fully expanded leaves were defined as Stage 2 and Stage 3, respectively. These leaves were green. The bottom leaves that began to become yellow were defined as Stage 4, and the leaves that became fully yellow or brown were defined as Stage 5.

To identify ladder-like DNA banding patterns, total DNA was extracted from the tip of leaves without the main vein using a modified CTAB method (Yamada et al. 2000) and then electrophoresed in a 2% agarose gel and stained with SYBR Gold nucleic acid gel stain (Molecular Probes, U.S.A.).

For TUNEL assay of leaf materials, the part that was near a vascular bundle of the leaf tip at each stage was fixed with 4% paraformaldehyde in PBS at 4°C (overnight), then embedded in Paraplast Plus embedding medium (Oxford, U.S.A.) and sectioned at 5 μm. The sections were attached to silane-coated microscope slides and dewaxed. TUNEL assay was carried out using the DeadEnd Fluorometric TUNEL System (Promega, U.S.A.) according to the manufacturer's instructions. All nuclei of the same sections were counterstained with propidium iodide.

Nuclear fragmentation was also detected by flow cytometric analysis (Yamada and Marubashi 2003). Leaves were chopped in 500 μl of ice-cold nuclear extraction buffer, which was included in the High Resolution Kit for Plant DNA (Partec, Germany). The extract was filtered through a 50 μm nylon mesh. The flow-through, including isolated nuclei, was collected and then 2.5 volumes of ice-cold nuclear staining buffer of the reagent set (Partec) was added and mixed well. The DNA content of the isolated nuclei was analyzed using a flow cytometer (Ploidy Analyzer, Partec). On the basis of histograms obtained from flow cytometry of a total of 20,000 nuclei, peak areas indicating fragmented nuclei (M1) and normal nuclei (M2) were determined, and then nuclear fragmentation rates (%) were calculated by the formula $\{(M1/M1+M2)\times 100\}$ using freeware (Win MDI version 2.8 software for flow cytometry analysis).

To detect morphological changes of nuclei and nuclear fragmentation in the cells of younger leaves (Stage 1, 2 and 3), protoplasts were isolated from the leaves at Stages 1 to 3 and observed under confocal laser scanning microscopy. The leaves were sectioned and treated with an enzyme solution containing 2% (w/v) Onozuka R-10 cellulase (Yakult Co., Japan), 0.2% (w/v) Macerozyme R-10 (Yakult Co., Japan), 0.7 M mannitol, and 10 mM $CaCl_2$, pH 5.6, for 3 hours at 30°C. The protoplasts were separated from cellular debris by filtering through a 53 μm nylon sieve. The protoplasts, stained with 0.5% DAPI, were observed under a confocal laser scanning microscope (Radiance 2100, Bio-Rad, U.S.A.) equipped with a blue diode laser (405 nm).

Agarose gel electrophoresis showed a ladder-like DNA banding pattern, which indicates internucleosomal fragmentation of nuclear DNA, and is a key feature of PCD (Figure 2). A ladder-like DNA banding pattern was detected not only in senescent leaves (Stages 4 and 5) but also in juvenile and young leaves (Stages 1, 2, and 3). The ladder pattern was very clear at Stage 1, became fainter gradually until Stage 3 and was clear again at Stages 4 and 5.

The TUNEL procedure also showed that DNA fragmentation occurred at juvenile and young stages (Figure 3). In the upper and lower epidermis, most of the nuclei were stained by TUNEL assay at Stages 1 and 2. At Stages 3, 4, and 5, the nuclei of the epidermis were no longer observed. In palisade cells and cells of the spongy layer, only a few nuclei were stained by TUNEL assay, and intact nuclei (TUNEL negative) visualized by counterstaining with propidium iodide were observed at Stage 1. At Stages 2, 3, 4, and 5, most of the nuclei in palisade cells and cells of the spongy layer were stained by TUNEL assay. Intact nuclei were observed at Stages 2 and 3, but were no longer observed at Stages 4 and 5. PCD of xylem was detected in Stage 1, but not detected

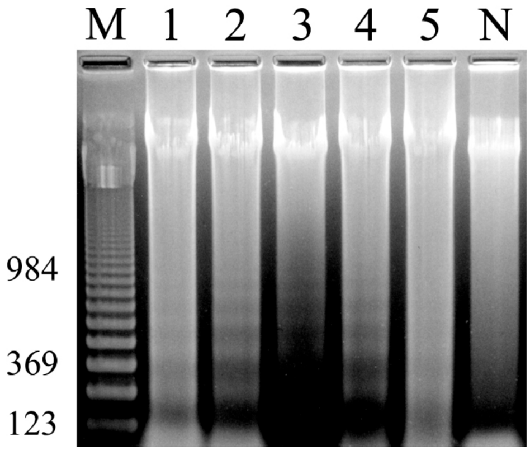

Figure 2. DNA fragmentation detected in the leaves by agarose gel electrophoresis. Total DNA was isolated from one leaf of each stage using a modified CTAB method, then electrophoresed in a 2% agarose gel and stained with SYBR Gold nucleic acid gel stain. All stages of leaf development generated DNA ladders, which indicate internucleosomal fragmentation of nuclear DNA. M: 123-bp DNA ladder marker; 1–5: Stages 1–5; N: negative control (suspension cultured cells of *N. tabacum*)

in Stages 2, 3, 4 or 5 by TUNEL assay (data not shown).

Flow cytometry yielded two peaks, likely to correspond to nuclei at the G_1 and G_2 phases of the cell cycle, from Stage 1 to 3. At Stages 4 and 5, the G_1 and G_2 peaks decreased, and additional peaks with a lower fluorescence value, indicating nuclear fragmentation, appeared (Figure 4). These additional peaks are one of the features of PCD (Yamada and Marubashi 2003).

Confocal laser scanning microscopy of protoplasts from juvenile and young leaves clarified the beginning stages of nuclear fragmentation. The nuclei of some protoplasts isolated from leaves at Stage 1 had lobes like the buds of yeast (Pinto et al. 2002) (Figure 5a). Nucleoli were not observed in nuclei that had lobes. The number of lobes was about one to three for a nucleus at Stage 1 (Figure 5b). At Stage 2, the number of nuclei with lobes and the number of lobes per nucleus increased (Figure 5c). At Stage 3, the lobes were separated from the nucleus. We named the material "nuclear dust" at this stage (Figure 5d). At Stage 3, various types of protoplasts were observed that had nuclei with lobes,

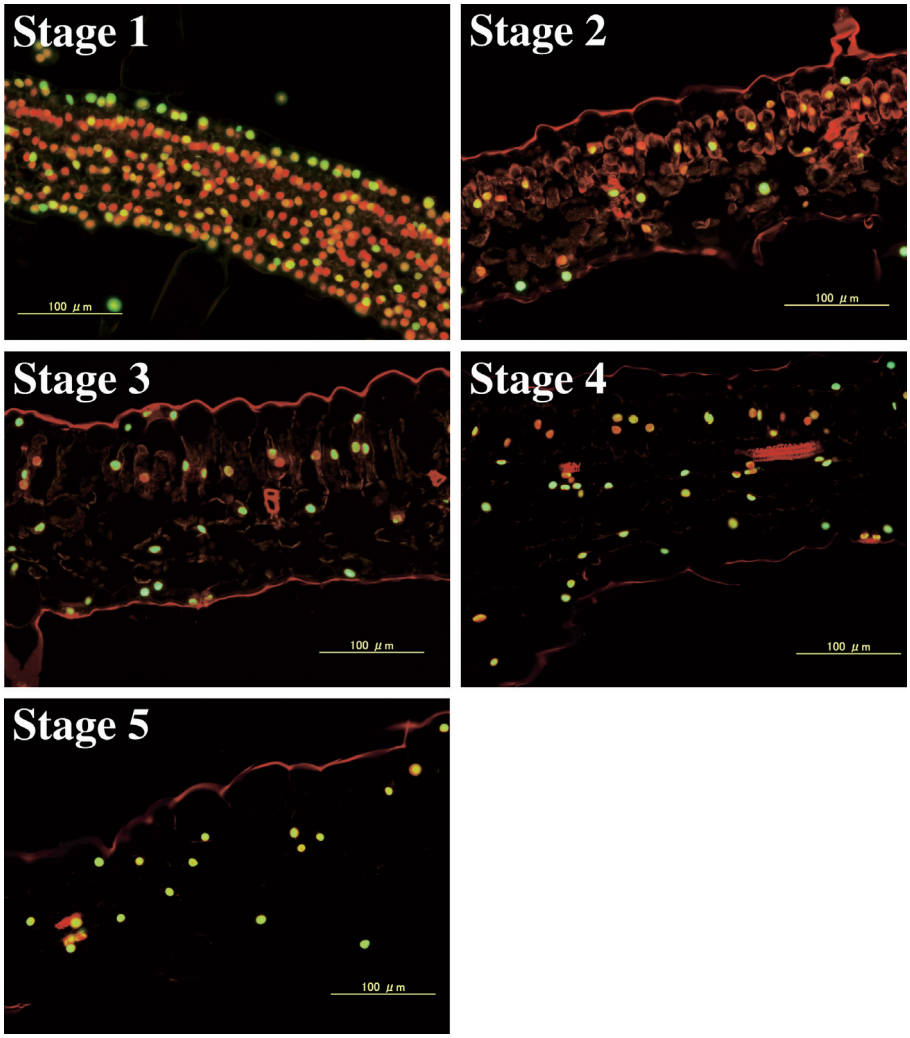

Figure 3. *In situ* detection of DNA fragmentation in leaves of tobacco. DNA fragmentation (green signals) was detected by the TUNEL procedure in all stages. Intact nuclei (red signals) were counterstained with propidium iodide.

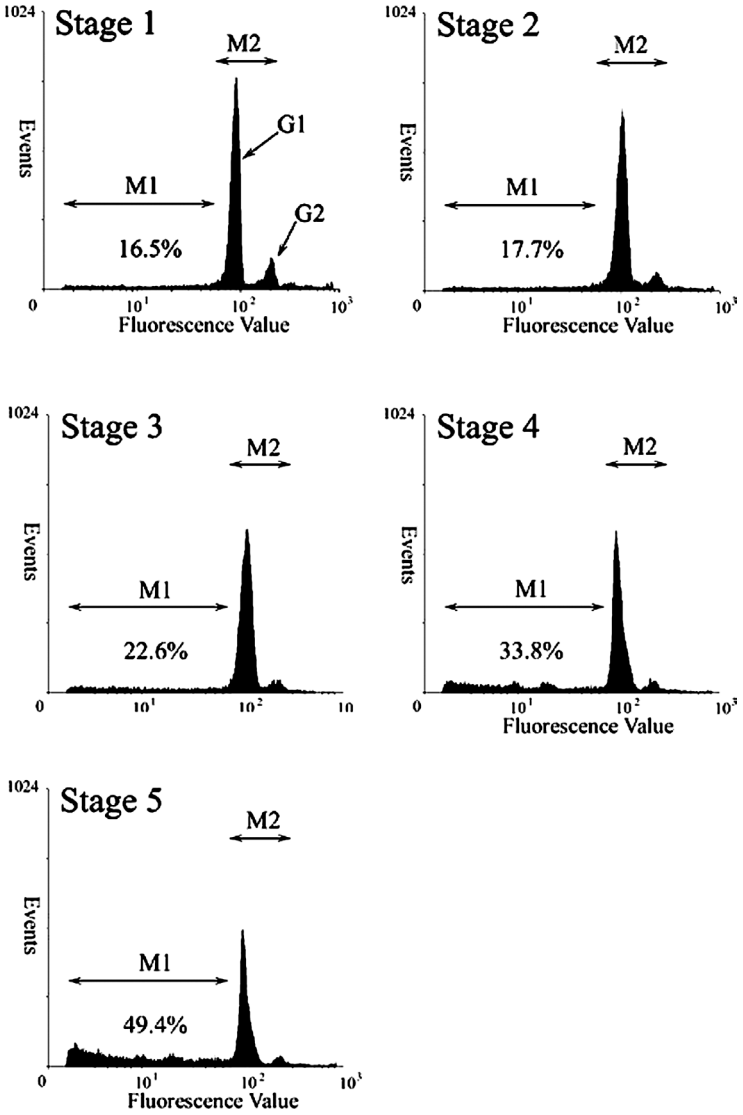

Figure 4. Nuclear fragmentation detected in the leaves. Histograms indicate an increase in fragmented nuclei (M1) and a decrease in normal nuclei (M2), as measured by flow cytometry of a total of 20,000 nuclei. The arrows indicate the peaks of nuclei, presumably corresponding to the G_1 and G_2 phases of the cell cycle.

nuclear dust or chromatin condensation. Because lobes and nuclear dust were stained by DAPI, they appear to consist of DNA. Though Pinto et al. (2002) reported that cultured tobacco BY-2 cells had nuclei with lobes when reactive oxygen species were added, nuclei with lobes have never been observed in tobacco plants during normal development.

Results of agarose gel electrophoresis revealed internucleosomal fragmentation of nuclear DNA, which is a key feature of PCD, not only at Stages 4 and 5 but also at Stages 1, 2, and 3. Moreover, the results of TUNEL assay showed DNA fragmentation, which is also a key feature of PCD, not only at Stages 4 and 5 but also at Stages 1, 2, and 3, while the results of flow cytometry indicated peaks of nuclear fragmentation, another feature of PCD, at Stages 4 and 5. Therefore, we confirmed that PCD occurred at Stages 4 and 5. These observations agree with those of Simeonova et al. (2000) that PCD is involved in leaf senescence. Although in the present study, a ladder-like DNA banding pattern was detected by agarose gel electrophoresis, peaks showing nuclear fragmentation were not detected by flow cytometry at Stages 1, 2 or 3. In these early stages, a nucleus with lobes might be counted as one nucleus by flow cytometry, moreover, most of the nuclei accompanied by fragmentation of nuclear DNA might not be recognized as fragmented nuclei by flow cytometry. We consider that a nucleus with lobes is at the beginning stages of nuclear fragmentation (Figure 5). Therefore, we conclude that PCD occurred not only in senescent leaves at Stages 4 and 5 but also in juvenile and young leaves at Stages 1, 2, and 3. Consequently, we conclude that even juvenile leaves of tobacco exhibit PCD and that tobacco leaves grow while undergoing PCD from the initial stages of

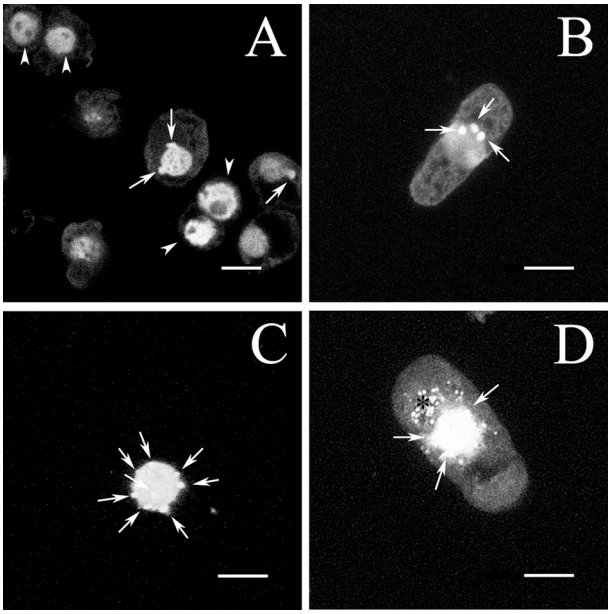

Figure 5. Confocal laser scanning micrographs of protoplasts isolated from the leaves of *N. tabacum* at Stages 1, 2, and 3. A: Some nuclei had one or two lobes (arrows) at Stage 1. Arrowheads indicate normal nuclei. B: A nucleus with three lobes at Stage 2. C: The number of lobes per nucleus increased at Stage 2. D: A nucleus with many lobes and nuclear dust (asterisk). Bars are 10 μm.

development until senescence.

The physiological implications of PCD in tapetum and nucellus are expected. Through tapetum cell death, the tapetal cells further contribute to completing the extracellular sculpting of the pollen grains, as well as providing them with adhesive and signaling molecules of proteinaceous and lipoidal nature that are critical for interacting with the pistil during pollination (Wu and Cheung 2000). The role of nucellar cell death is to create an intercellular space to ease the physical passage of the pollen tube in the direction of the female gametophyte (Hiratsuka et al. 2002).

The physiological meaning of PCD in the juvenile and young leaves is an important problem to be solved. Emptying of some epidermal cells by PCD might lead to better light transmission. Disappearance of some mesophyll cells by PCD might improve leaf gas exchange. At present, we infer that PCD in juvenile and young leaves is useful for improvement of photosynthetic efficiency.

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